5-2017

Effects of ultra-sonication process on digestibility of kafirin in sorghum

Sarah F. Beazley
University of Tennessee, Knoxville, sarahbeazley@mac.com

Effects of ultra-sonication process on digestibility of kafirin in sorghum

Sarah F. Beazley, Andrew C. Sullivan, Vermont P. Dia

Department of Food Science and Technology

College of Agricultural Sciences and Natural Resources

University of Tennessee, Knoxville

Abstract

The sorghum cereal crop has been noted for its ability to provide unique health benefits to the human species. It is the 5th most produced cereal in the world, and the United States is its top producer. The main shortcoming of this product is its low digestibility through the human digestive system. This study investigates a process to make the most abundant protein within sorghum, kafirin, more digestible. This was done by using a food process known as ultra-sonication, which introduces sound waves into a substance to promote agitation of particles. Through each analysis, ultra-sonication showed to positively affect kafirin's solubility by shortening protein fragments, improving hydrolysis, and significantly increasing its overall soluble protein content, and thus its digestibility.

1. Introduction

Not commonly considered a leading cereal crop, sorghum is nevertheless the fifth most produced cereal in the world (4). Sorghum bicolor L. Moench, generally referred to as *sorghum*, is native to Africa, where it still plays a large role in nourishing that population. In addition, the crop is popular in areas of Asia. These steppe climate zones complement sorghum production well, as it is drought resistant, unlike many other cereal crops (15). While these regions account for the majority of human consumption, the United States is currently the largest producer of sorghum, as it uses the crop for livestock feed (10, 15). Although not as popular in the U.S., sorghum has high potential for human health benefits. Sorghum products are a safe answer for people with coeliac disease, who are limited to gluten-free products and cannot consume wheat, rye, or barley (15).

Studies have tested the positive effects of compounds from sorghum on common diseases, including cancer (4, 13, 17). In addition, sorghum has had positive effects on diabetes, obesity, and other ailments (7, 9). Despite research highlighting the numerous positive effects of sorghum, one factor significantly hinders its success.

The cereal's main disadvantage stems from its inability to digest easily in the human gastric system (5). The indigestibility is due to sorghum's morphology, physicochemical composition, and protein interactions (10). In one study, it was found that sorghum had at least 20% less digestibility than rice, maize, and wheat (6). Further, the proteins within sorghum also account for this challenging feature. More digestible proteins possess higher nutritional value due to increased amino acid availability (5). Sorghum contains four main proteins: albumin, globulin, glutelin, and kafirin. The latter, kafirin, accounts for the majority of protein within sorghum and will be the focus of this research (3).

Kafirin's interactions with other proteins establish complexes, which in turn inhibit its solubility. One example of a specific inhibition would include an interaction with tannins (14). Tannins bind to proteins found in sorghum, and are suspected to be one explanation of why the kafirin protein is indigestible, as they are not found in other cereal crops including rice, wheat, and maize (7, 16). Although tannins do offer a beneficial antioxidant component, they have been shown to decrease digestibility, as studies have shown that sorghums containing tannins were more slowly digested than non-tannin sorghums (1, 2, 7, 16). Within kafirin, there are different conformations, each with different digestibility levels. The α

form is the most digestible, while the β and ϒ form contain much cysteine, which actually hinders the α form and thus, the digestibility (5).

These facts could logically lead to the question: How can one increase the digestibility of kafirin within sorghum? Enhancing its digestibility would allow higher nutritional value and produce more functionality for human health benefits (5). The prospect of improving digestibility and nutritional value of this grain, so prevalent in Africa and Asia, offers the potential to mitigate global food and nutritional needs in water-scarce regions of the world (11).

The hypothesis investigated and explained through this research is that kafirin solubility and nutritional benefit can be promoted through ultra-sonication. Ultra-sonication is a process that sends ultrasound waves, usually above 20 kHz, into a medium to create an agitation of said medium. This agitation could lead to cavitation in the substance, a formation of empty spaces within the matter. This thus could cause chemical changes, such as digestibility (8, 10).

The objective of this study was to isolate the protein kafirin from sorghum and establish how ultra-sonication affects its pepsin-pancreatin stability. If testing and analyses were to go as planned, the ultra-sonication of kafirin would impact the protein's pepsin-pancreatin digestibility beneficially.

2. Materials and Methods

2.1 Materials

Whole grain sweet white sorghum flour was obtained from Bob's Red Mill Natural Food, Inc. (Milwaukie, OR). The sorghum flour was defatted, and albumin,

globulin, and then kafirin protein was extracted from the sample, and it was subsequently distilled and freeze dried.

2.2 Bicinchoninic Acid Assay

This portion of the analysis measured protein concentration by Bicinchoninic Acid (BCA), and a standard curve was prepared before proceeding. For this, one vial of 2 mg bovine serum albumin (BSA)/mL solution was obtained and used to prepare standard solutions. The sample solutions were prepared by adding 1 mg of kafirin sample to 1-mL of 60% isopropanol, then vortexing 3k RPM at room temperature until the samples were fully dissolved. Next, a BCA working reagent (WR) was prepared by first calculating the total volume of WR needed, which was calculated to be 7380 µL. Next, 50 parts BCA Reagent A was mixed with 1 part BCA Reagent B, to equal the 7380 µL. To complete the assay, 25 µL of each sample was pipetted into a clear 96 well plate. As with all analyses in this study, this was completed in triplicate. Two hundred µL of the WR was added to each well. This was then mixed on a plate shaker for 30 seconds. The plate was covered with parafilm and incubated at 37°C for 30 minutes, followed by cooling to room temperature. At this point, the absorbance was taken of the samples at 490 nm with a microplate reader and soluble protein concentration calculated from BSA standard curve.

2.3 Molecular weight profile

This analysis separated the molecules according to their molecular mass (in kDa), using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The sample was prepared by dissolving 1 mg/mL of sample in 60%

isopropanol, then vortexing at 3K RPM at room temperature until the sample was fully dissolved. The sample was then analyzed by SDS-PAGE under reducing conditions. Twenty µL of sample was added to each well of a 4-20% Mini-Protean TGX Gel (Bio-Rad Laboratories, Hercules CA). The electrophoresis was run at 180 V for 35 minutes. Fifty mL of Coomassie blue dye was added and incubated overnight at 4°C. The sample was destained in deionized water until a clear background is obtained.

2.4 Orthophtaldialdehyde Degree of Hydrolysis

This analysis measured degree of hydrolysis with orthophtaldialdehyde (OPA). First, a solution was prepared containing 25 mL of sodium tetraborate solution, 2.5 mL of 20% sodium dodecyl sulfate solution, and 200 µL of OPA. Using deionized water, the volume was adjusted to 50 mL. The reagent was stored in the dark until ready for use. Ten µL of sample in 60% isopropanol (1 mg/mL) was combined with 3.4 mL of the OPA reagent in a 96 well plate. After letting stand for 2 minutes at 25°C, the absorbances were measured at a wavelength of 340 nm. From this, the degree of hydrolysis was calculated using Equation 1.

$$DH(\%) = \frac{ABS * 1.934 * d}{c} \quad (1)$$

2.5 Trichloroacetic Acid Soluble Protein Content

Three hundred µL aliquots of the hydrolysate sample were combined with 300 µL of 20% of trichloroacetic acid (TCA) solution. This was used to find the soluble and insoluble fractions in 10 % TCA. This was then allowed to stand for 30 minutes at room temperature. The mixture was then centrifuged for 10 minutes at 4°C and 3000xG. The supernatant was then analyzed for soluble protein content

using the BCA Assay method of analysis. Then, using Equation 2, the degree of hydrolysis was calculated.

$$DH(\%) = \frac{\text{soluble protein content in 10 g\% TCA (mg)}}{\text{total protein content (mg)}} * 100 \quad (2)$$

2.6 Hydrochloric Acid Soluble Protein Content

This analysis of protein content uses the same methodology as the TCA analysis with some minor changes. Three hundred µL aliquots of the hydrolysate sample were combined with 300 µL of 20% of hydrochloric acid (HCl) solution. This was used to find the soluble and insoluble fractions in 10 % HCl. This was then incubated at 100°C for 4 hours. The mixture was then centrifuged for 10 minutes at 4°C and 3000xG. The supernatant was then analyzed for soluble protein content using the BCA Assay method of analysis. The degree of hydrolysis was then calculated using Equation 2.

2.7 Statistical Analysis

All analyses were run in triplicate. Statistical Analysis of data was run by the PROC GLM of Statistical Analysis System software version 9.4 using Tukey's posthoc with a 95% confidence interval.

3. Results

3.1 Bicinchoninic Acid Assay

The protein concentrations of the kafirin solutions were calculated by their measured absorbance through BCA assay **(Fig. 1).** Ultra-sonication at 20% amplitude decreased protein concentration from 464.7 µg/mL (no ultra-sonication) to 449.7 µg/mL after 5 minutes and 370.7 µg/mL after 10 minutes

(Fig. 1). **Fig. 1** also shows that ultra-sonication at 40% amplitude decreased protein concentration. Although slight, after 5 minutes at 40% amplitude, the protein concentration dropped from 464.7 μg/mL to 463.0 μg/mL. After 10 minutes, the protein concentration dropped from 464.7 μg/mL to 386.4 μg/mL.

3.2 SDS-PAGE

This analysis was conducted to confirm the correct protein product was being tested after extraction from the sorghum flour **(Fig. 2)**. The three bands that can be seen in **Fig. 2** represent the three different conformations of kafirin: α, β, and Ɣ.

3.3 Ortophtalaldehyde Degree of Hydrolysis

The OPA degree of hydrolysis was calculated through **Eq. 1** using measured absorbances of the samples **(Fig. 3)**. Overall, the degree of hydrolysis was increased by any ultra-sonication **(Fig. 3)**. At 20% amplitude, the degree of hydrolysis of the sample was raised from 1.000 to 1.153 after 5 minutes, but only barely raised to 1.003 after 10 minutes **(Fig. 3)**. **Fig. 3** also shows that at 40% amplitude, ultra-sonication increased the degree of hydrolysis to 1.198after 5 minutes and 1.178 after 10 minutes.

3.4 Trichloroacetic Acid Soluble Protein Content

The soluble protein content in TCA was determined by using the BCA method of analysis **(Fig. 4)**. As seen in **Fig. 4**, the soluble protein content was increased by ultra-sonication. At 20% amplitude of ultra-sonication, the soluble protein content was raised from 168.8 μg/mL to 230.5 μg/mL after just 5 minutes, and to 237.4 μg/mL after 10 minutes **(Fig. 4)**. At 40% amplitude, the soluble protein content

increased to 306.6 µg/mL after 5 minutes and to 347.2 µg/mL after 10 minutes **(Fig. 4)**.

3.5 Hydrochloric Acid Soluble Protein Content

Similar to the TCA soluble protein content, but instead measuring the hydrolysis of all protein content, the HCl soluble protein content was also measured using the BCA method of analysis **(Fig. 5)**. In this case, ultra-sonication decreased the soluble protein content **(Fig. 5)**. **Fig. 5** shows that at 20% amplitude, the HCl soluble protein content dropped from 546.3 µg/mL to 420.2 µg/mL after 5 minutes and to 358.6 µg/mL after 10 minutes. After 5 minutes at 40% amplitude, the soluble protein content decreased to 384.1 µg/mL and after 10 minutes at 40% amplitude it decreased slightly less, to 425.8 µg/mL **(Fig. 5)**.

4. Discussion

Protein concentration is directly proportional to absorbance of a solution. Through the BCA assay method, the absorbance was measured with a microplate reader at a 490 nm wavelength. **Fig. 1** shows that there is no significant difference between the solutions that were and were not ultra-sonicated, except for one solution. Only the protein concentration ultra-sonicated for 10 minutes at 20% amplitude was significantly lower than its starting concentration. This data suggests that principally, the waves of ultra-sonication broke down the protein to smaller amino acid chains and peptide portions, making the substance more soluble.

This gel electrophoresis analysis confirmed that the protein extracted was, in fact, kafirin. The α, β, and ϒ confirmations of the protein can all be found as

bands on **Fig. 2**, organized top to bottom from heaviest to lightest molecular weight in kDa. With the correct protein confirmed, the experiment could move forward as planned.

The OPA degree of hydrolysis analysis also involved the use of calculation from measured absorbance. **Fig. 3** shows that ultra-sonicated solutions all had significantly higher degrees of hydrolysis but one: 20% amplitude after 10 minutes. All others (20% amplitude after 5 minutes and 40% amplitude after both 5 and 10 minutes) produced significantly higher degrees of hydrolysis than the starting point **(Fig. 3)**. The degree of hydrolysis is proportionally the cleaved peptide bonds in a protein hydrolysate (12). An increase in degree of hydrolysis translates to an increase in digestibility. Combined with this data, this definition offers that ultra-sonication increases degree of hydrolysis of kafirin, and thus increases the cleavage of peptide bonds within the protein.

This analysis tests for the measurable nitrogen component of the soluble protein content before and after ultra-sonication. From all the analyses run, this showed the most significant results. **Fig. 4** shows clearly how as ultra-sonication increases in both time and amplitude, so does the amount of soluble protein content. Amplitude at both 20% and 40% after both 5 and 10 minutes all produced significantly higher soluble protein content than the kafirin solution with no ultra-sonication **(Fig. 4)**. The TCA converts amides into amines and carboxylic acid, and the protein content measured was the amine portion. The sound waves disrupted the bonds around the digestible conformation of kafirin, and increased the protein's solubility.

This analysis is very similar to the previous, but instead tested for total soluble protein content. Instead of showing increased protein content, the HCl analysis indicates that ultra-sonication decreases that content **(Fig. 5)**. Though contrasting, these results are still significantly different than the starting content and show a significant decrease once ultra-sonication starts. The results are less steady though, as the 20% amplitude created less soluble protein content than the 40%. Rather than breaking down the amide portions of the protein, the HCl solution broke down the protein in its entirety. However, the ultra-sonicated kafirin was already somewhat disassembled, and so the prolonged exposure to HCl (4 hours) in turn made the protein contain a decreased amount of soluble protein content.

5. Conclusion

In closing, the objective of this experiment was to determine the effects of ultra-sonication on different aspects of the kafirin protein, and the results did show positive effects on each analysis run. The BCA assay displayed that ultra-sonication decreased protein concentration, which means that the process successfully produced smaller peptide fragments and amino acid chains, as they measure less protein concentration. The OPA test showed that the degree of hydrolysis was increased by ultra-sonication, and thus more easily solubilized. The TCA soluble protein content measurement showed that the ultra-sonication process significantly increased the solubility of kafirin with each increase in amplitude and time, suggesting that the process positively affected kafirin's digestibility.

The prospect of improving digestibility and nutritional value of a cereal native to water-scare regions of the world is profound, as populations in these regions continue to increase, as climate-related weather changes continue to affect the planet, and as global food supply concerns remain. The health benefits of sorghum on disease can also be greatly improved by an increase in digestion, and would profit from this and future research on this area. Further exploration of these conclusions could include future studies focusing on how to further increase kafirin's digestibility. Similar research involving a different process to alter the morphological structure and/or properties of kafirin or other sorghum protiens would be the next logical step in the progression to improving its solubility.

6. Appendix

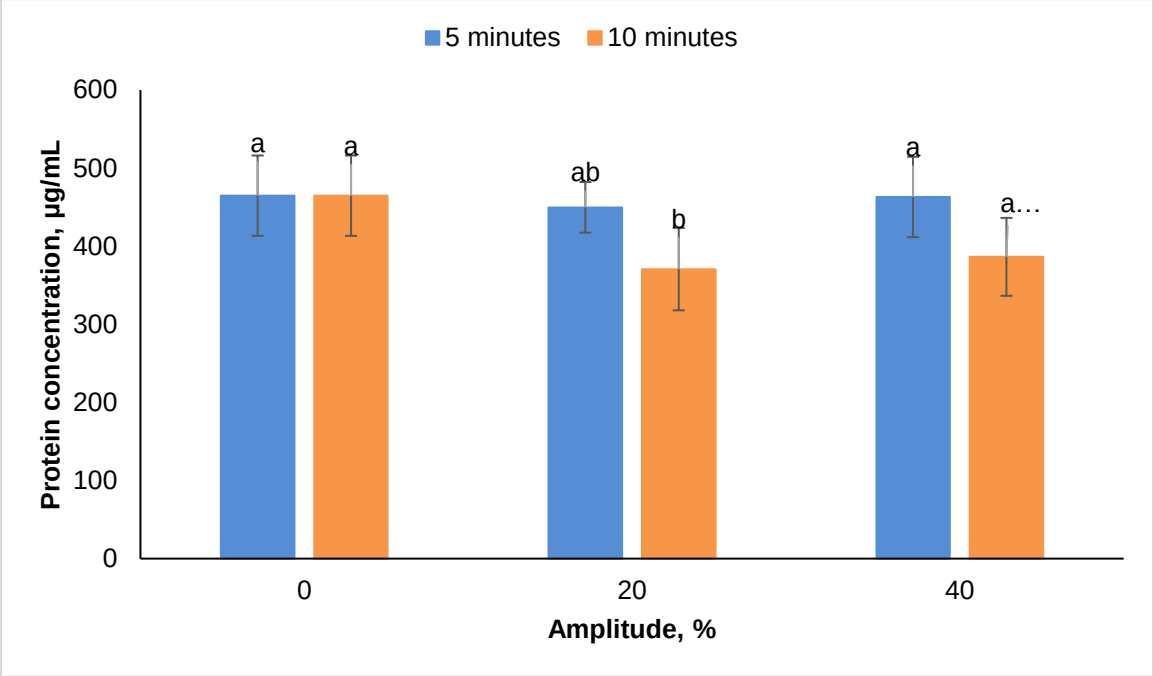

Figure 1. BCA Assay results showing the effect of ultra-sonication on protein concentration of kafirin after 5 minutes and 10 minutes at both 20% and 40% amplitude.

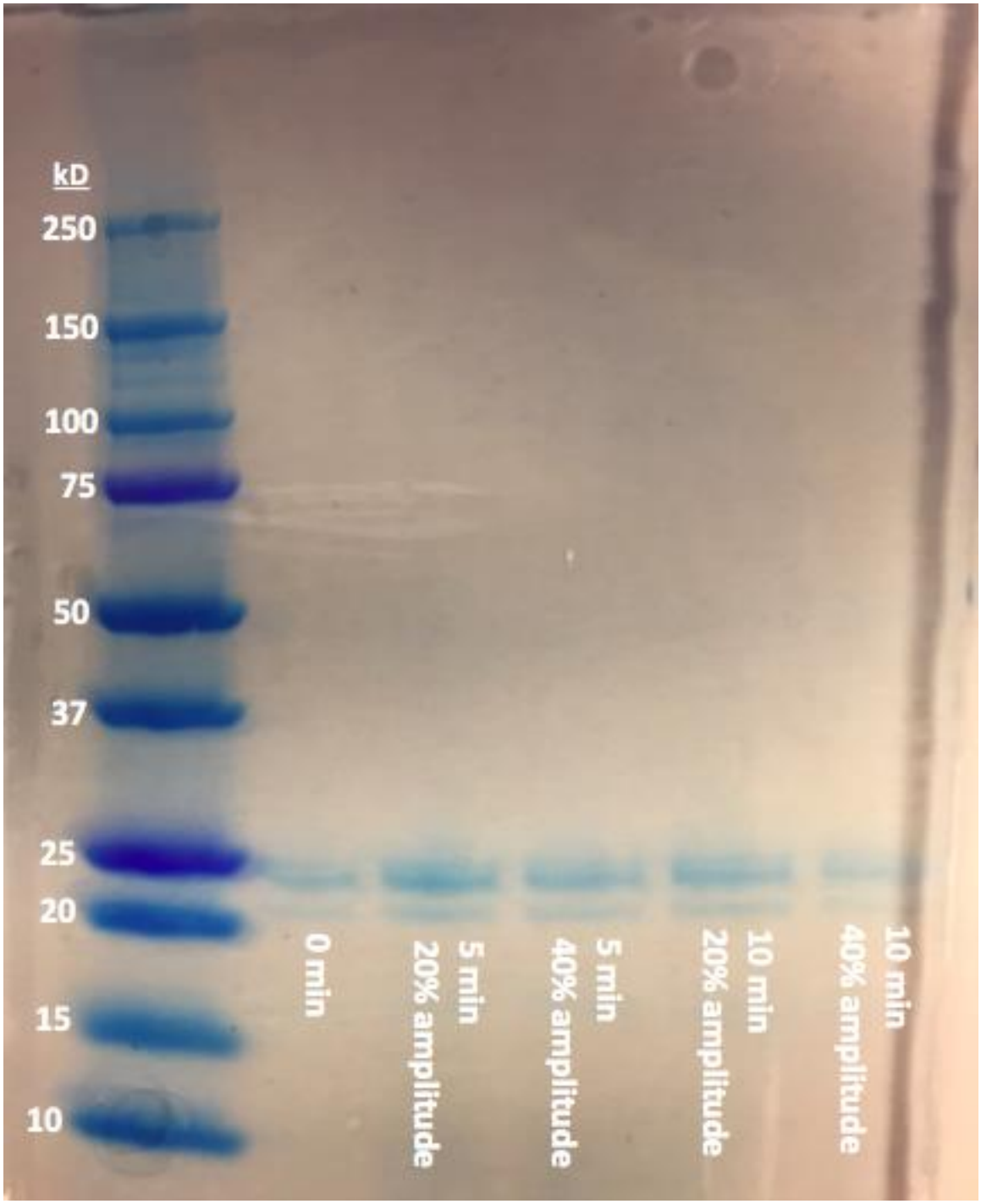

Figure 2. SDS-PAGE results displaying the molecular mass of each kafirin solution and conformation after ultra-sonication for 5 and 10 minutes at 20% and 40% amplitude.

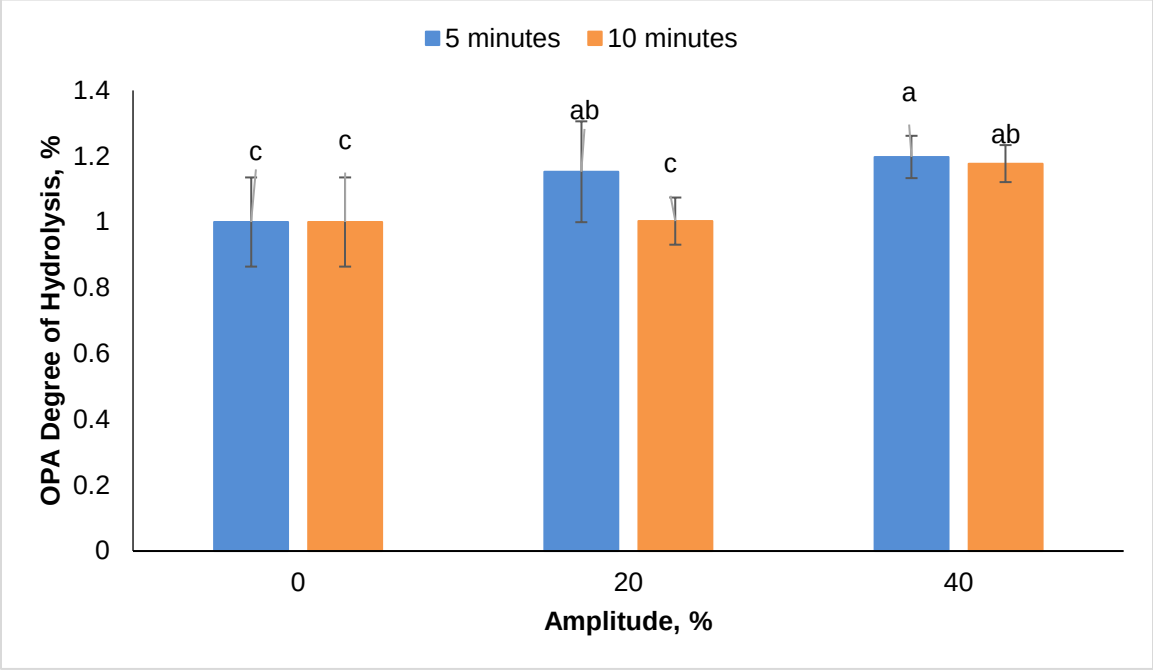

Figure 3. OPA results showing the effect of ultra-sonication on degree of hydrolysis of kafirin after 5 and 10 minutes at 20% and 40% amplitude.

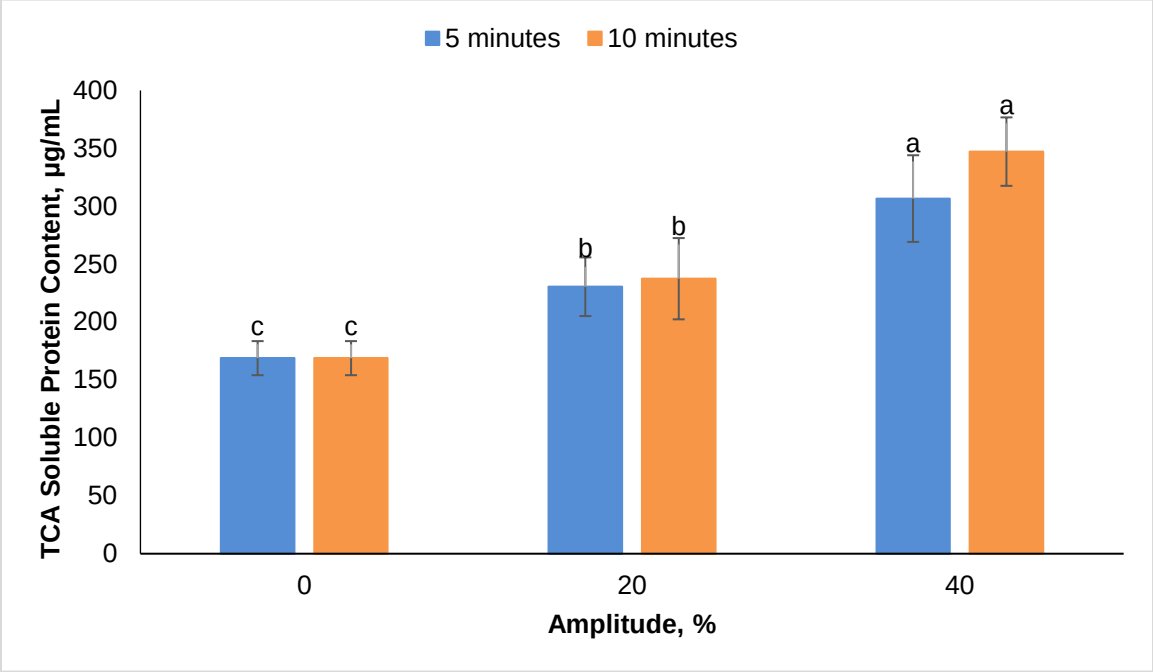

Figure 4. TCA results showing the increase of soluble nitrogen protein content from the ultra-sonication of kafirin after 5 and 10 minutes at 20% and 40% amplitude.

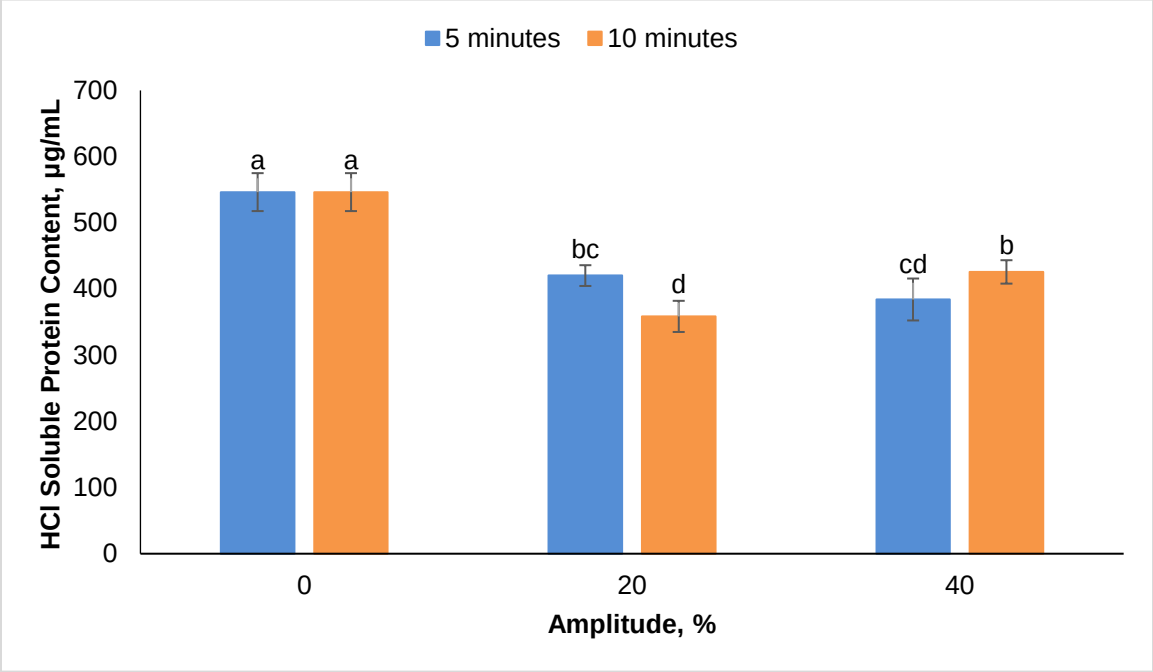

Figure 5. HCl results showing the effect of ultra-sonication on total soluble protein content after 5 and 10 minutes at 20% and 40% amplitude.

References

1. Chung, K., Wong, T. Y., Wei, C., Huang, Y., Lin, Y. (1998). Tannins and Human Health: A Review. *Critical Reviews in Food Science and Nutrition, 38*(6), 421-464.
2. De Castro Palomino Siller, A. In Vitro Starch Digestibility Estimated Glycemic Index of Sorghum Products. M.Sc. Thesis, Texas A&M University, College Station, TX, USA, 2006.
3. De Mesa-Stonestreet, N. J., Alavi, S., Bean, S. R. (2010). Sorghum Proteins: The Concentration, Isolation, Modification, and Food Applications of Kafirins. *Journal of Food Science, 75*(5), R90-R104.
4. de Morais Cardoso, L., Pinheiro, S. S., Martino, H. S. D., Pinheiro, S., & Ana, H. M. (2017). Sorghum (Sorghum bicolor L.): Nutrients, bioactive compounds, and potential impact on human health. *Critical Reviews in Food Science and Nutrition, 57*(2), 372-390.
5. Duodu, K. G., Taylor, J. R. N., Belton, P. S., Hamaker, B. R. (2003). Factors affecting sorghum protein digestibility. *Journal of Cereal Science, 38*(2), 117-131.
6. Hamaker, B. R., Kirleis, A. W., Butler, L. G., Axtell, J. S., Mertz, E. T. (1987). Improving the *in vitro* protein digestibility of sorghum with reducing agents. *Proceedings of the National Academy of Sciences of the United States of America*, *84*, 626-628.
7. Lemlioglu-Austin, D., Turner, N. D., McDonough, C. M., Rooney, L. W. (2012). Effects of Sorghum [*Sorghum bicolor* (L.) Moench] Crude Extracts on Starch Digestibility, Estimated Glycemic Index (EGI), and Resistant Starch (RS) Contents of Porridges. *Molecules, 17*(9), 11124-11138.
8. Mason, T. J. (1988). Sonochemistry: Uses of Ultrasound in Chemistry and Related Disciplines. *Developments in Cardiovascular Medicine, 178*, 25-54.
9. Moraes, E. A., da Silva Marineli, R., Lenquiste, S. A., Steel, C. J., de Menezes, C. B., Queiroz, V. A. V., Maróstica, M. R. (2015). Sorghum flour fractions: Correlations among polysaccharides, phenolic compounds, antioxidant activity and glycemic index. *Food Chemistry, 180*, 116-123.
10. Park, S. H., Bean, S. R., Wilson, J. D., Schober, T. J. (2006). Rapid Isolation of Sorghum and Other Cereal Starches Using Sonication. *Cereal Chemistry, 83*(6), 611-616.
11. Rao, P. P., Birthal, P., Reddy, B. V., Rai, K., & Ramesh, S. (2006). Diagnostics of sorghum and pearl millet grains-based nutrition in India. *International Sorghum and Millets Newsletter, 47*, 93-96.
12. Rutherfurd, S. M. (2010). Methodology for determining degree of hydrolysis of proteins in Hydrolysates: a review. *Journal of AOAC INTERNATIONAL, 93*(5), 1515-1522.
13. Shih, C.-H., Siu Ng, R., Wong, E., Chiu, L. C. M., Chu, I. K. and Lo, C. (2007). Quantitative analysis of anticancer 3-deoxyanthocyanidins in infected sorghum seedlings. *Journal of Agricultural and Food Chemistry,* 55(2), 254-259.

14. Talyor, J., Bean, S. R., Ioerger, B. P., Taylor, J. R. N. (2007). Preferential binding of sorghum tannins with γ-kafirin and the influence of tannin binding on kafirin digestibility and biodegradation. *Journal of Cereal Science, 46*, 22-31.
15. Taylor, J. R. N., Schober, T. J., Bean, S. R. (2006). Novel food and non-food uses for sorghum and millets. *Journal of Cereal Science, 44*(3), 252-271.
16. Wu, Y., Li, X., Xiang, W., Zhu, C., Lin, Z, Wu, Y., Li, J., Pandravada, S., Ridder, D. D., Bai, G., Wang, M. L., Trick, H. N., Bean, S. R., Tuinstra, M. R., Tesso, T. T., Yu, J. (2012). Presence of tannins in sorghum grains is conditioned by different natural alleles of *Tannin1*. *Proceedings of the National Academy of Sciences of the United States of America, 109*(26), 10281-10286.
17. Yang, L., Browning, J. D. and Awika, J. M. (2009). Sorghum 3-deoxyanthocyanins possess strong phase II enzyme inducer activity and cancer cell growth inhibition properties. *Journal of Agricultural and Food Chemistry, 57*(5),1797–1804.